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Structure and homology of human C1q receptor (collectin receptor)

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SUMMARY

In this paper we report partial amino acid sequence for C1q receptor (C1qR). The N-terminal amino acid sequence of isolated C1qR and the sequences of peptides obtained by V8/trypsin digestion show a high degree of similarity to the cDNA-derived amino acid sequence of a human protein which was initially reported as a component of RoSSA and subsequently as calreticulin. This sequence in turn shows homology with *Onchocerca volvulus* antigen (RAL-1) and B50 murine melanoma antigen. A component of approximately 53,000 MW, isolated from human spleen, was found to have identical mobility on SDS-PAGE to C1qR and identical N-terminal sequence, but a different overall charge. Human antibodies from Sjögren's syndrome patients did not recognize C1qR, but showed positive reaction with the purified 53,000 MW component from spleen. Rabbit antibodies against denatured C1qR, in contrast, recognized both C1qR and the purified 53,000 MW component. The 53,000 MW spleen component thus has an identical N-terminal sequence to calreticulin, and to the reported RoSSA component, and is recognized by antibodies in Sjögren's syndrome sera. The data obtained indicate that C1qR and the reported calreticulin/RoSSA component are similar but not identical molecules, which belong to the same protein superfamily.

INTRODUCTION

The existence of a cell surface receptor for the C1q complement component was first suggested by Dickler and Kunkel.¹ C1q receptor (C1qR) activity has been reported on most leucocytes, endothelial cells, fibroblasts and platelets² and binding of C1q to its receptor has been reported to mediate a range of phenomena, including phagocytosis, modulation of cytokine and immunoglobulin secretion, and polymorphonuclear leucocyte-endothelium interaction.³ Erdei and Reid⁴ extracted radioactive C1qR from surface-radioiodinated U937 cells and in biosynthetic labelling studies using [³⁵S]cysteine and [³⁵S]methionine they showed that C1qR is synthesized by U937 cells. The molecular species identified as C1qR by Erdei and Reid⁴ has been purified from human tonsil lymphocytes, U937 cells and human spleen.^{5,6} C1qR purified from these sources is a protein of molecular weight 56,000, as assessed by SDS-PAGE under reducing conditions. C1qR is an acidic glycoprotein with 15-20% carbohydrate⁶ and the detergent-solubilized protein behaves as an elongated dimer of molecular weight

115,000 ± 7000. Purified C1qR was found to inhibit the binding of radioiodinated C1q to U937 cells.⁷ Three other proteins, conglutinin, lung surfactant protein A and mannan-binding protein, were found to interact with purified C1qR.⁷ These three proteins, like C1q, have been reported to be involved in phagocytosis and have similar structural organization to that of C1q.⁸ The interaction of these four ligands with C1qR indicates that their opsonic activity may arise through interaction of these proteins with C1qR. Due to the structural and functional similarities between conglutinin, lung surfactant protein A, C1q and mannan-binding protein and the complement association or carbohydrate-binding characteristics of these ligands, we earlier proposed the name collectins for the ligands of C1qR,⁹ and the term collectin receptor for C1qR. Although the binding characteristics and cellular distribution of C1qR are documented and possible physiological roles of C1qR have been reported by a number of workers, no primary structure data have been reported in the past. In this paper we present peptide sequence for the collectin receptor and report on the sequence homology of C1qR with other proteins.

MATERIALS AND METHODS

Purification of C1qR

C1qR was purified from tonsil lymphocytes by the method of Malhotra and Sim,⁶ except that the sample was further processed on a TSKgel DEAE-NPR (4.6 × 35 mm) high-perfor-

Abbreviations: BSA, bovine serum albumin; C1qR, C1q receptor; PBS, phosphate-buffered saline: 8.2 mM Na₂HPO₄/1.5 mM KH₂PO₄ buffer, pH 7.5, containing 139 mM NaCl and 3 mM KCl.

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mance liquid chromatography (HPLC) column (TOSHAAS, Philadelphia, PA), pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The bound protein was eluted with a 20-ml linear gradient (flow rate, 1 ml/min) of 0–500 mM NaCl in Tris-HCl buffer (pH 8.0). This step removed detergent as well as removing minor contaminants. Purification of C1qR was monitored by a functional assay (ligand binding) as described previously.^{6,7} C1qR was radioiodinated using a standard Lodogen reaction.¹⁰

Purification of 'RoSSA'

Human spleen (50 g) was homogenized in 50 ml of 0.01 M sodium phosphate buffer (pH 7.2) containing 150 mM NaCl. The homogenate was centrifuged at 40,000 *g* for 2 hr and RoSSA antigen was purified from the supernatant by the method of Wu *et al.*¹¹ The material was further purified using high-pressure gel ion-exchange filtration, exactly as described for C1qR above, to isolate a homogeneous component of approximately 53,000 MW, termed by us 'RoSSA'.

To determine whether C1qR could be purified from the same source, the pellet from the homogenized spleen was subjected to detergent extraction. The pellet was redissolved in 10 mM phosphate buffer (pH 7.4), containing 1% (w/v) Nonidet P-40, 100 µg of soya bean trypsin inhibitor/ml, 5 mM iodoacetamide, 2.5 mM di-isopropyl phosphorofluoridate, 20 µM 1,10-phenanthroline and 5 µg of pepstatin/ml. The detergent-soluble material was loaded on to a 1-ml Mono Q fast protein liquid chromatography (FPLC) column (Pharmacia), pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.4), containing 0.1% (w/v) emulphogene BC720 (Sigma, Poole, U.K.). Bound proteins were eluted with a linear gradient of 0–1 M NaCl as described previously.⁶ The C1qR pool from Mono Q was then separated from minor contaminants on a TSKgel DEAE-NPR HPLC column, as described earlier.

Large-scale partial purification of crude RoSSA

Larger quantities of human RoSSA were purified from approximately 200 g of human spleen following the method of Venables *et al.*¹² Fractions containing RoSSA antigen were identified by counter-current immunoelectrophoresis. This material was used to make RoSSA-Sepharose resin for adsorption of antibody preparations. Subsequent analysis of the material on a TSKgel DEAE-NPR HPLC column, as above, showed that it did not contain component 2 identified in Fig. 3.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as described by Laemmli.¹³ Samples were prepared (reduced or/and alkylated) as described by Fairbanks *et al.*¹⁴ Proteins were detected with Coomassie Brilliant Blue.¹⁴

Immunization of rabbits

Rabbits were injected in the popliteal node region with C1qR (20 µg) in polyacrylamide slices excised from SDS-PAGE. The acrylamide was homogenized and mixed with 1 ml of complete Freund's adjuvant (CFA). Three booster injections each of 60 µg of soluble purified C1qR in 1 ml of incomplete Freund's adjuvant (IFA) were given over a period of 3 months.

Purification of antibodies

Anti-C1qR immunoglobulins were purified from pooled rabbit antiserum by triple sodium sulphate precipitation.¹⁵ Purified antibody was dialysed against phosphate-buffered saline (PBS).

Sjögren's syndrome sera

Human sera positive for anti-RoSSA were obtained from patients with primary Sjögren's syndrome. Sera were analysed for antibody against RoSSA and LaSSB at the Danish State Serum Institute by the standard technique involving Ouchterlony immunodiffusion against extract of human spleen (RoSSA) or extract of rat thymus (LaSSB), and involving suitable positive control standard sera. The sera selected for use in the present study showed positive reaction with RoSSA.

Immunoblotting (Western blot analysis)

Proteins to be immunoblotted were run on SDS-PAGE under non-reducing conditions, and transferred to nitrocellulose (Hybond C, Amersham International, Amersham, U.K.). Antigens were detected using first antibodies and second antibody-alkaline-phosphatase conjugates. The blot was developed as described by Kölbl *et al.*¹⁶

Immunoassay (antigen capture assays)

Microtitre plate wells were coated with 100 µl of purified rabbit anti-C1qR antibody (50 µg/ml) in PBS and non-specific binding sites were blocked with bovine serum albumin (BSA) (3 mg/ml) in PBS. Different dilutions of C1qR (50 µl; max conc. 50 µg/ml) in PBS were mixed with 50 µl of ¹²⁵I-labelled C1qR (2.5 µg/ml, total c.p.m. loaded/well 1.2 × 10⁶) and added to the antibody-coated wells. BSA (50 µl; 50 µg/ml) along with radiolabelled C1qR were loaded on the plate as a control. The plate was incubated for 2 hr at ambient temperature (18–22°C). The plate was washed extensively with PBS. Bound radioactivity was solubilized and removed with 300 µl of 4 M NaOH.

Agglutination of U937 cells

U937 cells were grown in 50-ml culture flasks under standard conditions.¹⁷ Cells (~10⁷) were washed three times with PBS and were suspended in 1 ml of PBS. The cell pellet obtained from 200 µl of this cell suspension was resuspended in 200 µl of purified anti-C1qR immunoglobulin (0.7 mg/ml) in PBS containing 0.1% (w/v) sodium azide and incubated for 30 min at ambient temperature. Immunoglobulin (0.7 mg/ml) purified from the serum of the rabbit prior to immunization was used as a negative control. Cells were washed three times with 1 ml of PBS containing 0.1% (w/v) NaN₃. To the cell pellet was added 100 µl of goat anti-rabbit IgG (500 µg/ml) in PBS containing 0.1% (w/v) NaN₃ and the cell suspension was incubated for 30 min at ambient temperature. After extensive washing in PBS the cells were suspended in 1 ml of PBS. The cell suspension was observed for agglutination by light microscopy.

Fluorescence detection of bound antibodies

U937 cells (~10⁷) were washed, resuspended and treated with anti-C1qR immunoglobulin as described earlier. Immunoglobulins (0.7 mg/ml) purified from the serum of the rabbit prior to immunization or purified rabbit antibody raised against complement component C3 was used as a negative control. Cells were washed three times with 1 ml of PBS/NaN₃. After washing three times with PBS the cells were incubated with anti-rabbit

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immunoglobulins conjugated with fluorescein isothiocyanate (FITC) (100 μ l; diluted in PBS/NaN₃ according to the value recommended by the manufacturer: Serotec, Kidlington, U.K.). FITC-labelled cells were observed and photographed by fluorescence microscopy using a Zeiss Axioskop microscope.

Coupling of partially purified RoSSA to CNBr-activated Sepharose 4B and fractionation of anti-C1qR antibodies

Coupling of partially purified RoSSA to CNBr-activated Sepharose 4B (Pharmacia, Milton Keynes, U.K.) was carried out in 50 mM potassium phosphate buffer (pH 8.2) by the method described by the manufacturer. Protein, 1.5 mg, was coupled per ml of Sepharose. The resin was washed twice with five volumes of coupling buffer followed by a wash with 50 ml of 1 M NaCl. Unreacted binding sites were blocked with 0.1 M ethanolamine-HCl buffer (pH 8.5). RoSSA-Sepharose 4B (3.5 ml) was pre-equilibrated in PBS and poured into a column. Rabbit anti-C1qR antiserum (1 ml) was loaded on to the affinity column, and the column was washed with PBS, until the OD₂₈₀ of the eluate was reduced to background. The column was washed with 6 ml of deionized water and the bound-IgG was eluted with 3 M MgCl₂. The material which did not bind to the RoSSA-Sepharose was pooled and designated as 'C1qR-specific' antiserum, whereas the fractions eluted with 3 M MgCl₂ were designated as 'RoSSA cross-reactive' antibodies.

Reduction and alkylation of proteins

Samples for sequence analysis were denatured by the addition of 7.5 M guanidium chloride, 0.125 M Tris-HCl (pH 8.0) and reduced by the addition of dithiothreitol at a final concentration of 6.5 mM. The samples were incubated for 1 hr at 37°, then 4-vinylpyridine was added to a final concentration of 88 mM to block free cysteine residues. The samples were further incubated for 1 hr at 37°, dialysed exhaustively against 10 mM NH₄OH, and freeze dried.

Amino acid sequencing

Purified C1qR was reduced and alkylated and the N-terminal amino acid sequence was obtained using an Applied Biosystems 470A protein sequencer and Applied Biosystems 120A analyser. C1qR was found to be highly resistant to trypsin digestion. Therefore, the protein was digested with *Staphylococcus aureus* V8 protease (BCL, Lewes, U.K.). Purified C1qR (200 μ g) was reduced and alkylated and dialysed against 0.1% (w/v) ammonium bicarbonate. The protein was incubated with 5% (w/w) V8 protease for 2 h at 37°. The peptides were separated on a C₈ reverse-phase HPLC column, pre-equilibrated in 0.1% trifluoroacetic acid (TFA). Peptides were eluted with an acetonitrile gradient (0–100%) in 0.1% TFA. The amino acid composition of purified peptides was analysed by HPLC with the Waters Pico-Tag system (Millipore, Watford, U.K.) after hydrolysis in 6 M HCl (6 hr at 125°). Appropriate peptides were subjected to sequence analysis as above.

In a second set of experiments purified C1qR (50 μ g) was reduced and alkylated and dialysed against 0.1% (w/v) ammonium bicarbonate. The protein was incubated with 2 μ g of V8 protease for 16 hr at 37°, followed by 16 hr incubation at 37° with 2 μ g of TPCK-treated trypsin. The peptides generated were purified on a Vydac C₄ HPLC column (250 \times 4.6 mm), pre-equilibrated in 0.1% TFA. Peptides were eluted with a linear gradient of 4–72% acetonitrile in 0.1% TFA.

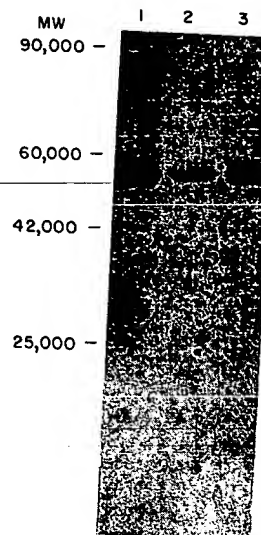


Figure 1. Analysis by SDS-PAGE of different 53,000 MW components isolated from spleen and tonsils. These were run on a 10% gel, and are non-reduced. The major components are all of apparent molecular weight 53,000. Lane 1, RoSSA purified by the method of Wu *et al.*¹¹ without the further purification described in Materials and Methods; lane 2, component 2 from the RoSSA preparation, found to co-run with C1qR on the TSKgel DEAE-NPR HPLC column; lane 3, C1qR purified from tonsils.

RESULTS

N-terminal sequence of C1qR

C1qR was purified as described in Materials and Methods. Purified material, as analysed on SDS-PAGE, under non-reducing conditions, is shown in Fig. 1 (lane 3). A sequence of 14 amino acids at the N-terminal of C1qR was obtained (Table 1). This sequence was found to be identical to the N-terminal sequence of a protein reported to be a component of the autoantigen RoSSA¹⁸ (Table 1).

Peptide sequence

To obtain peptides for sequencing, purified C1qR was digested with *Staphylococcus aureus* V8 protease or with V8 protease and trypsin. Nine of the total of 12 peptides sequenced had sequences identical to segments of the reported RoSSA component^{18,19} (Table 1). Two of the remaining three peptides show similarity to the RoSSA component¹⁹ and the other peptide sequence is not present in the reported sequence of the RoSSA component¹⁹ (Table 1). As noted in Table 1, there is clear quantitative evidence that the peptides which do not match the RoSSA sequence are not derived from contaminants.

Specificity of anti-C1qR antibodies

The specificity of antisera raised against C1qR was established by radioimmunoassay and by Western blot analysis. Radioimmunoassay was performed as described in Materials and Methods. Different dilutions of unlabelled C1qR and BSA were used as potential inhibitors for binding of radioiodinated C1qR to anti-C1qR antibodies. Concentration-dependent inhibition of binding of ¹²⁵I-labelled C1qR to microtitre plate-bound anti-

Table 1. Amino acid sequences of C1qR peptides. Except for the sequences obtained for peptides 1, 2 and 3, all the other peptide sequences are identical to segments of the reported RoSSA component.¹⁹ On a quantitative basis peptides 1 and 2 (yield at first sequence step 70 pm each; not present in RoSSA) were isolated in the same quantity as peptide 6 (initial yield 79 pm; present in the RoSSA sequence); all the three peptides were generated by V8 digest and were from the same batch of digest. This is a strong indication that peptides 1 and 2 are not derived from contaminants

Peptide no.	Peptide sequence	Sequence identity or similarity with RoSSA (region)
N-terminal of C1qR	EPAYVFKEQFLDGD	position 1-14
Peptides generated by V8 protease digest		
1	YKGRQT	Two KG-QT sequences in RoSSA
2	DNQSENMS	not present in RoSSA
3	KPADMS-S	KPEDWDPE sequence present in RoSSA
4	QFLDGDG-TS	position 9-18
5	IDNPE	position 278-282
6	DNPEYSPDPSIYAYDNFVDL-L	position 279-300
7	PDPSIYAYDNFVDL	position 285-298
Peptides generated by Trypsin/V8 protease digest		
8	KPEDWDEEMDGEWEPPVIQNP	position 233-253
9	GLDLWQVK	position 299-306
10	IDNPEYSPDPSIY	position 278-290
11	VKIDNSQVE	position 168-176
12	FTVK	position 79-82

C1qR was observed in the presence of unlabelled purified C1qR (Fig. 2a), whereas BSA did not affect the binding of radiolabelled C1qR to the antibody. This confirms the recognition of ¹²⁵I-labelled C1qR and unlabelled C1qR by the antibody preparation. Partially purified C1qR from detergent-soluble material from the spleen (after Mono Q FPLC column chromatography) was separated by SDS-PAGE (Fig. 2b; lane 1) and blotted on nitrocellulose filters as described in Materials and Methods. The blots were probed with anti-C1qR antiserum and rabbit anti-C3 antiserum as well as with immunoglobulin from the control preimmunization bleed of an anti-C1qR rabbit. A single band corresponding to C1qR was recognized by the anti-C1qR serum (Fig. 2b, lane 2), but no band with similar MW was recognized by the control antisera (not shown).

Purification of RoSSA

C1qR was found to have a high degree of primary sequence identity with a protein reported initially to be one of the components of the complex RoSSA autoantigen¹⁹ (Table 1). Subsequent work²⁰ has indicated that the cDNA sequence presented by McCauliffe *et al.*¹⁹ may correspond to human calreticulin, and there is further uncertainty as to whether the cDNA sequence does represent a component of RoSSA.²⁰ The function and the cellular localization of the proteins (RoSSA component or calreticulin) encoded by the cDNA sequence^{19,20} are uncertain. C1qR, in contrast, has been isolated on the basis of a highly defined function^{5,6} and is expressed on the cell surface. However, C1qR is clearly closely related to the cDNA-defined sequence.^{19,20} In order to investigate further the relationship between C1qR and the related species, RoSSA was partially purified from a spleen extract by a standard method.¹¹ Wu *et al.*¹¹ reported isolation of two polypeptides with MW of

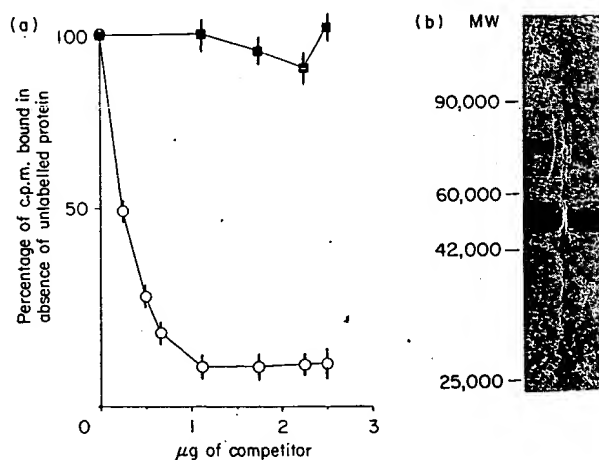


Figure 2. Specificity of anti-C1qR antibodies. (a) Binding of radiolabelled C1qR to solid phase immobilized anti-C1qR antibodies in the presence of unlabelled C1qR or BSA. Different dilutions of unlabelled C1qR (O) were mixed with radiolabelled C1qR in PBS and loaded on to microtitre plate wells coated with purified anti-C1qR antibody. BSA (□) mixed with radiolabelled C1qR was loaded as a control. After extensive washing the bound c.p.m. were measured, as described in Materials and Methods. Control antibodies, namely purified rabbit antibodies against C3 and immunoglobulins purified from serum of the rabbit prior to immunization with C1qR, did not recognize radioiodinated C1qR (not shown). Results of a single experiment with the average and range of triplicate experimental points are shown. (b) Western blot analysis of partially purified C1qR with anti-C1qR antibody. Partially purified C1qR from detergent solubilized extract from spleen was separated by SDS-PAGE and blotted on nitrocellulose filters. Lane 1, Coomassie Blue-stained gel track; lane 2, blot probed with purified anti-C1qR antibody. A single band corresponding to C1qR was detected.

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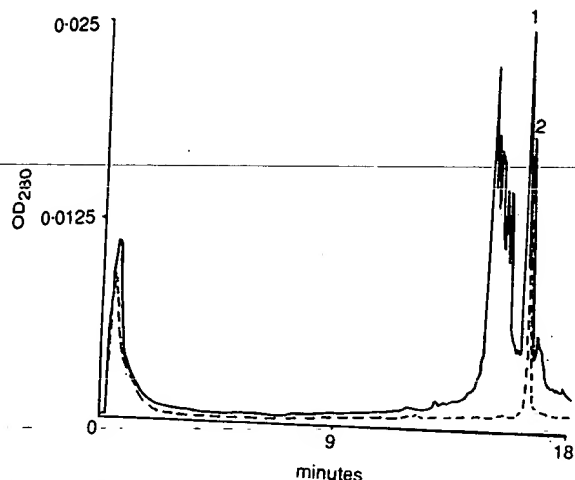


Figure 3. Elution profile of purified C1qR (---) from tonsils and partially purified RoSSA (—) from the TSKgel DEAE-NPR HPLC column. Only peaks 1 and 2 contained material of apparent molecular weight-53,000 (unreduced).

approximately 60,000 and 55,000, as analysed on SDS-PAGE under reducing conditions. Both these molecules were reported to react with human anti-RoSSA antibodies. Using the same method, an apparent single major polypeptide of MW 53,000 (Fig. 1, lane 1) under non-reducing conditions was obtained by us, which showed positive reaction in Western blotting with anti-RoSSA serum. C1qR also migrates as a 53,000 MW protein on SDS-PAGE under non-reducing conditions. The fractions containing RoSSA antigen were further purified on the TSKgel DEAE-NPR HPLC column as described in Materials and Methods. The 53,000 MW material was found to separate into two components of apparently identical MW, component 1 (major) and 2 (minor) (Fig. 3), on the HPLC column. The minor component, component 2 (Fig. 1, lane 2), was found to co-elute with C1qR activity (Fig. 3), while the other was less acidic. The N-terminal sequence (up to 12 residues) of both the components was found to be identical to the N-terminal sequence of C1qR purified from tonsils (Table 1), which in turn is identical to the reported N-terminal sequences of a RoSSA component^{18,19} and calreticulin.²⁰ Of these two components, only component 1 was recognized by human antibodies in Sjögren's syndrome sera. We therefore interpret component 1 as being an appropriate candidate for a component of RoSSA autoantigen, while component 2 is C1qR, as determined by functional assays.⁵⁻⁹ Component 1 is referred to hereafter as 'RoSSA'.

Purification of C1qR from detergent solubilized spleen lysate

The pellet obtained from the spleen homogenate after the standard RoSSA extraction method was redissolved in detergent-containing buffer and the procedure for purification of C1qR was followed. Analysis of partially purified C1qR on SDS-PAGE under non-reducing conditions is shown in Fig. 2b (lane 1). C1qR from spleen migrated identically to tonsil C1qR on the TSKgel DEAE-NPR HPLC column (Fig. 3).

Western blot analysis and antibody binding of 'RoSSA' and C1qR

The results above indicate that C1qR has considerable but not

complete sequence identity to the RoSSA component reported by McCauliffe *et al.*¹⁹ and has the same N-terminal sequence as an anti-RoSSA reactive protein (Fig. 3, component 1) isolated by us from spleen. However, C1qR does not co-elute with 'RoSSA' on the TSK-DEAE HPLC column, and can be differentially extracted from spleen. To investigate the similarities between C1qR and 'RoSSA', Western blot analysis of the different molecules of apparent MW 53,000 (non-reducing) isolated from spleen and tonsils was performed. The proteins were blotted onto nitrocellulose. Different sets of filters were treated with Sjögren's syndrome sera from any of eight patients or with anti-C1qR antiserum and the blots were developed with alkaline phosphatase conjugated anti-human IgG and anti-rabbit IgG respectively. The results of the experiments are shown in Table 2. All the 53,000 MW proteins showed positive reaction with anti-C1qR antibody, whereas antibodies from Sjögren's syndrome sera only interacted with purified 'RoSSA' (component 1). To investigate further the antigenic differences between C1qR and 'RoSSA', anti-C1qR antiserum was fractionated on a RoSSA-Sepharose 4B affinity column made with partially purified RoSSA. Purified C1qR and 'RoSSA' were separated on SDS-PAGE and were blotted on nitrocellulose filters. Different sets of filters were incubated with unfractionated anti-C1qR, and with the putative C1qR-specific and RoSSA cross-reactive fractions obtained from RoSSA-Sepharose. Unfractionated anti-C1qR, C1qR-specific and RoSSA cross-reactive material showed positive reaction with C1qR (Table 2), whereas C1qR-specific showed no reaction with 'RoSSA' (Table 2). Therefore the anti-C1qR antiserum can successfully be absorbed to remove specificity for 'RoSSA' without complete removal of anti-C1qR activity.

To establish further the differences between C1qR and 'RoSSA', the binding of purified C1qR and 'RoSSA' to solid phase immobilized anti-RoSSA immunoglobulins (i.e. the immunoglobulin fraction from Sjögren's syndrome sera), anti-C1qR immunoglobulins and control immunoglobulins was investigated. Microtitre plate wells were coated with 100 μ l of purified rabbit anti-C1qR antibodies (50 μ g/ml) or purified human anti-RoSSA antibodies (50 μ g/ml) or purified rabbit anti-ovalbumin antibodies (50 μ g/ml) in PBS. Non-specific binding sites were blocked with BSA (3 mg/ml) in PBS. ¹²⁵I-labelled C1qR or ¹²⁵I-labelled 'RoSSA' in PBS was loaded on to the immunoglobulin-coated wells and incubated for 24 hr at 4°. After extensive washings with PBS the bound c.p.m. were measured as described in Materials and Methods. The results of the binding experiments are shown in Fig. 4. Radioiodinated 'RoSSA' was found to bind both to anti-C1qR and to anti-RoSSA, whereas radioiodinated C1qR did not bind significantly to anti-RoSSA. The Western blot analysis and the solid phase binding studies indicate that C1qR and 'RoSSA' have antigenic differences.

Agglutination of U937 cells by anti-C1qR antibodies

McCauliffe *et al.*¹⁹ stated, without presenting experimental data, that the antibodies raised against the amino terminal portion of the protein encoded by their cDNA sequence (a sequence segment identical to the N-terminus of C1qR), locate only an intracellular species in mouse L cells (fibroblasts) and human Hep-2 cells (epithelioid cells). Erdei and Reid⁴ however, showed, by surface radioiodination of U937 cells, that C1qR is located

Table 2. Cross-reactivity between RoSSA and C1qR. Western blot analysis of different 53,000 MW components isolated from spleen and tonsils with anti-C1qR antiserum and anti-RoSSA antiserum. Materials blotted were components 1 and 2 from the spleen RoSSA preparation, isolated C1qR from tonsils (co-running with component 2 on HPLC) and the C1qR extract from spleen after the FPLC mono Q step of purification

Antiserum	Blotted protein			
	RoSSA component 1 from spleen	C1qR (tonsils)	Partially purified C1qR (detergent solubilized spleen extract)	Component 2 from the spleen RoSSA preparation
Anti-RoSSA antiserum (human)	+++	---	---	---
Antiserum to denatured C1qR (rabbit)	+++	+++	+++	+++
Antiserum to denatured C1qR (rabbit), not bound to RoSSA-Sepharose.	---	+++	ND*	ND
Antiserum to denatured C1qR (rabbit), which bound to RoSSA-Sepharose	+++	+++	ND	ND

* ND, not done.

on the cell surface. Purified soluble C1qR also inhibits the binding of radiolabelled C1qR to U937 cells,⁷ providing further evidence for the surface localization of C1qR. To examine further the cellular localization of C1qR, U937 cells were treated with purified anti-C1qR antibodies as described in Materials and Methods. In the presence of anti-C1qR strong aggregation of U937 cells was observed (Fig. 5a), whereas in the presence of non-immune rabbit immunoglobulins, the cells were not agglutinated (Fig. 5b). In a second set of experiments U937 cells were treated with anti-C1qR antibody or non-immune rabbit immunoglobulins, followed by interaction with FITC-labelled anti-rabbit IgG. Figure 5c shows that in the intact cells specific

plasma membrane immunofluorescence was observed. This confirms that the anti-C1qR antibodies are recognizing an antigen exposed on the cell surface.

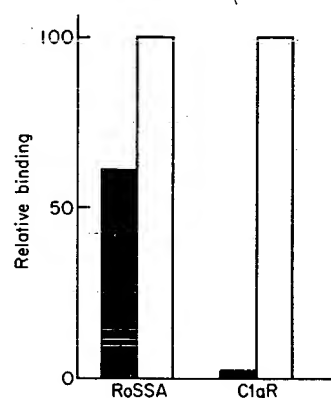


Figure 4. Binding of radioiodinated C1qR or radioiodinated 'RoSSA' to immobilized anti-C1qR antibodies or anti-'RoSSA' antibodies. Radioiodinated C1qR (c.p.m. loaded/well 1×10^6 ; specific activity 9×10^7 c.p.m./ μ g) or radioiodinated RoSSA (component 1 from HPLC (c.p.m. loaded/well 1×10^6 ; specific activity 9×10^7 c.p.m./ μ g) was interacted with immobilized purified antibodies against C1qR (\square), 'RoSSA' (\blacksquare) for 24 hr at 4°. After extensive washing bound c.p.m. were measured, as described in Materials and Methods. Binding is measured relative to the number of c.p.m. bound in the C1qR-anti-C1qR interaction. Results are the average of duplicate experiments. Background binding of radiolabelled ligand to purified anti-ovalbumin-coated wells has been subtracted.

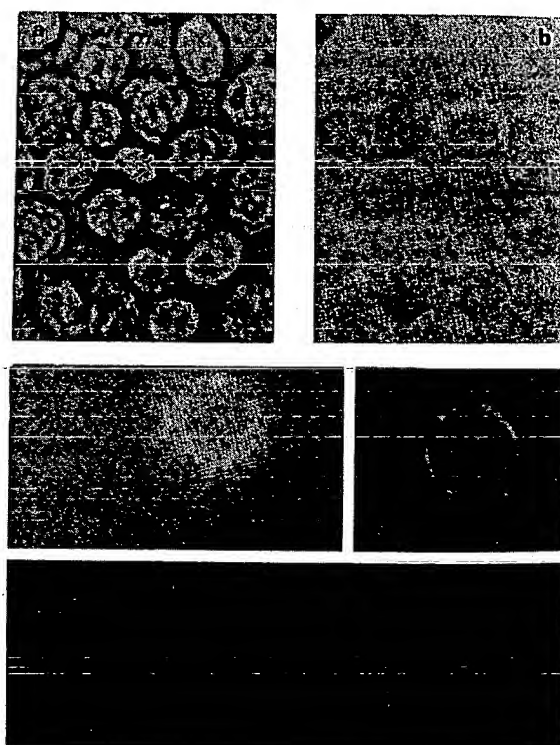


Figure 5. Agglutination of U937 cells by anti-C1qR antibodies. U937 cells (10^7 cells) were incubated with purified anti-C1qR antibodies and control antibodies in PBS containing NaN₃, as described in Materials and Methods. (a) Agglutination of U937 cells in the presence of purified anti-C1qR antibodies; (b) U937 cells in the presence of control non-immune IgG; (c) immunofluorescence staining of unfixed U937 cells. Unfixed U937 cells were treated with first and second antibody in the presence of NaN₃ at 4°, as described in Materials and Methods. Cells exposed to anti-C1qR antibody showed specific staining of the plasma membrane. Results of several different experiments are shown. The cells treated with control serum were not stained and were not visible in these conditions.

In this description, general 1, 2 and portions of the sequence that C molecules quantify not may have 1 peptidic derived 1 through exchange are not the Ro out to comes on the case a contains C C1qR present suggest presentations, c determine significant higher quantitate under: good a The ce describe data pr the cell two pr studies. RoSSA

Human ClqR	E P A V Y F K E Q F L D G D G - T S
Murine Calreticulin	D P A I Y F K E Q F L D G D A W T N
Murine B50 melanoma antigen	- - A I Y F K E Q F L N G N A
Human RoSSA/calreticulin	E P A V Y F K E Q F L D G D G W T S
Rat protein 425	- P A - Y F - E Q F L D - R A
Aplysia p407 (Aplysia californica snail neuronal protein 407)	- P T V Y F K E E F G D R A E

Figure 6. Amino-terminal sequence of ClqR and its homology with RoSSA/calreticulin,^{19,20} mouse calreticulin,²² murine B50 melanoma antigen,²³ rat protein 425²⁴ and aplysia protein p407.²⁴ The amino acids not present in the ClqR sequence are underlined.

DISCUSSION

In this paper the partial amino acid sequence of ClqR is described. In Table 1 the sequences of the ClqR peptides generated by different methods are shown. Except for peptides 1, 2 and 3, all the peptides show complete sequence identity with portions of the reported calreticulin²⁰ or RoSSA component sequences.¹⁹ The protein sequence obtained for ClqR indicates that ClqR and the cDNA-defined component^{19,20} are related molecules with a high level of sequence similarity. On a quantitative basis (Table 1) the peptides from ClqR which do not match the RoSSA/calreticulin cDNA-derived sequence have been sequenced at the same quantitative level as the peptides matching the cDNA-derived sequences, and are derived from the same digests. Since ClqR has been purified through multiple steps, ending with a very high resolution ion exchange, this strongly suggests that the non-matching peptides are not from contaminants, and therefore that the sequence of the RoSSA component/calreticulin^{19,20} is not identical throughout to that of ClqR. Further evidence to support this view comes from the composition analysis. McCauliffe *et al.*¹⁹ suggest on the basis of neuraminidase, endo- α -N-acetylgalactosaminidase and glycopeptidase digestion that the RoSSA protein contains no carbohydrate, although the cDNA sequence indicates one potential N-linked glycosylation site. Analysis of ClqR reported earlier,⁶ however, shows that glucosamine is present, and the carbohydrate composition of ClqR strongly suggests that two or three N-linked carbohydrate chains are present on each molecule.⁶ Comparison of amino acid compositions, compiled from the cDNA sequence¹⁹ and experimentally determined for ClqR,⁶ shows considerable similarities, but with significant differences. It is notable that the cysteine content is higher in ClqR (3%) than in RoSSA (1%). Experimental quantitation of cysteine in amino acid analysis generally leads to underestimation, but for ClqR, cysteine was measured, with good agreement, as both an oxidized and an alkylated form.⁶ The cellular localization of the RoSSA antigen component described by McCauliffe *et al.*¹⁹ appears to be intracellular. The data presented in this paper and elsewhere show that ClqR is on the cell surface. Further evidence for non-identity between the two proteins was provided by the antigenic cross-reactivity studies. Anti-ClqR antibodies were found to cross-react with 'RoSSA' (i.e. the species isolated by us from spleen), but

Sjögren's syndrome patients sera with anti-RoSSA antibody showed no reaction with ClqR. The rabbit anti-ClqR antibodies preabsorbed with RoSSA-Sepharose showed positive reaction with ClqR and no reaction with RoSSA. On the basis of cellular localization, composition and antigenicity, therefore, it appears that ClqR is not identical to the protein/cDNA described by McCauliffe *et al.*¹⁹ or Rokeach *et al.*,²⁰ but rather is a closely related protein with segments which are identical to those of the calreticulin/RoSSA protein. The presence of extensive identical and non-identical segments in proteins is an unusual type of homology, but as noted by McCauliffe *et al.*,¹⁹ the *Onchocerca volvulus* RAL-1 antigen²¹ shows this type of relationship to the cDNA-derived RoSSA/calreticulin sequence. The *O. volvulus* protein is 63% identical to the RoSSA component over the region of sequence available for comparison, but contains segments of completely identical, and segments of completely different sequence. A group of proteins, many of which are characterized as calcium-binding proteins, have a similar N-terminal sequence to ClqR and to calreticulin/RoSSA component. Some of these are shown in Fig. 6. The complete sequence of the human calreticulin/RoSSA component is very closely related (92% identity) to the sequences of mouse and rabbit calreticulin,²² as might be expected if it is a species homologue. Among the proteins shown in Fig. 6, it is of interest that two mouse proteins, melanoma antigen B50 and calreticulin, have very similar but not identical N-terminal sequences, but the reported cellular localization of the two molecules is different. B50 antigen has been reported on the cell surface of non-melanoma and melanoma cell lines,²³ whereas calreticulin has been reported as an endoplasmic protein.²² The relationship between two mouse proteins may resemble that between the two human proteins, ClqR and the calreticulin/RoSSA species.

The interpretation of the relationship of ClqR to the reported RoSSA¹⁹ or calreticulin²⁰ cDNA sequence is made complex by controversy as to whether the cDNA sequence does indeed represent a RoSSA component, or whether it represents calreticulin. Calreticulin is not thought to be recognized by autoantibodies in Sjögren's syndrome sera.²⁰ However in this study, we have isolated by high resolution chromatographic methods a homogeneous protein species from spleen which has the same N-terminal sequence as the reported RoSSA component,^{18,19} and is recognized by antibodies in Sjögren's syndrome

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sera which have been characterized, by standard methods, as RoSSA positive. To this extent, our results are in agreement with McCauliffe *et al.*¹⁹ We have also isolated a homogeneous protein, ClqR with the *same* N-terminal sequence, which is *not* recognized by Sjögren's syndrome antibodies, which has internal sequence which is different from the cDNA-derived RoSSA or calreticulin sequence. The discussion in ref. 19 on the similarity between RAL-1 antigen and the RoSSA/calreticulin sequence, and the data presented in Fig. 6, suggest that there is a group of very closely related proteins, which exhibit an unusual type of homology, with blocks of identical sequence, and blocks of completely unrelated sequence. If this is the case, then the use of cDNA sequencing, particularly polymerase chain reaction (PCR)-based cDNA, without extensive use of direct confirmation by protein sequence will not serve to distinguish between closely related proteins.

In conclusion, the similarity in polypeptide size, overall charge and in blocks of amino acid sequence between ClqR, the reported human RoSSA component, calreticulin, *O. volvulus* RAL-1 antigen, murine B50, aplysia p407 and rat 0425, indicate that ClqR is part of a highly conserved protein family.

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